Paracrine or virus-mediated induction of decorin expression by endothelial cells contributes to tube formation and prevention of apoptosis in collagen lattices

Elke Schönherr[1)a], Brian C. O'Connell[b], Johannes Schittny[c], Horst Robenek[d], Dirk Fastermann[a], Larry W. Fisher[b], Gabriele Plenz[d], Peter Vischer[d], Marian F. Young[b], and Hans Kresse[a]

[a] Department of Physiological Chemistry and Pathobiochemistry, University of Münster, Münster/Germany
[b] National Institute of Dental Research, National Institutes of Health, Bethesda, MD/USA
[c] Department of Anatomy, University of Berne, Berne/Switzerland
[d] Department of Arteriosclerosis Research, University of Münster, Münster/Germany

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Resting endothelial cells express the small proteoglycan biglycan, whereas sprouting endothelial cells also synthesize decorin, a related proteoglycan. Here we show that decorin is expressed in endothelial cells in human granulomatous tissue. For in vitro investigations, the human endothelium-derived cell line, EA.hy 926, was cultured for 6 or more days in the presence of 1 % fetal calf serum on top of or within floating collagen lattices which were also populated by a small number of rat fibroblasts. Endothelial cells aligned in cord-like structures and developed cavities that were surrounded by human decorin. About 14 % and 20 % of endothelial cells became apoptotic after 6 and 12 days of co-culture, respectively. In the absence of fibroblasts, however, the extent of apoptosis was about 60 % after 12 days, and cord-like structures were not formed nor could decorin production be induced. This was also the case when lattices populated by EA.hy 926 cells were maintained under one of the following conditions: *1)* 10 % fetal calf serum; *2)* fibroblast-conditioned media; *3)* exogenous decorin; or *4)* treatment with individual growth factors known to be involved in angiogenesis. The mechanism(s) by which fibroblasts induce an angiogenic phenotype in EA.hy 926 cells is (are) not known, but a causal relationship between decorin expression and endothelial cell phenotype was suggested by transducing human decorin cDNA into EA.hy 926 cells using a replication-deficient adenovirus. When the transduced cells were cultured in collagen lattices, there was no requirement of fibroblasts for the formation of capillary-like structures and apoptosis was reduced. Thus, decorin expression seems to be of special importance for the survival of EA.hy 926 cells as well as for cord and tube formation in this angiogenesis model.

[1)] Dr. Elke Schönherr, Department of Physiological Chemistry and Pathobiochemistry, University of Münster, Waldeyerstr. 15, D-48149 Münster/Germany, e-mail: schonhe@uni-muenster.de, Fax: ++2518355596.

Introduction

The growth of new capillaries by a process of sprouting from an established blood vessel has been termed angiogenesis. Angiogenesis is a complex phenomenon that is regulated by the interplay of a variety of factors that leads to the proliferation of endothelial cells, the formation of cords and finally to the cessation of growth and lumen formation (see [14, 36, 37] for reviews). The necessary changes in gene expression are driven both by cytokines such as vascular endothelial growth factor (VEGF), fibroblast growth factors (in particular FGF-1/aFGF and FGF-2/bFGF) and platelet-derived endothelial cell growth factor (PD-ECGF), as well as by components of the extracellular matrix.

The formation of sprouts during angiogenesis requires the migration of endothelial cells through an extracellular matrix, a process that requires the secretion of matrix-degrading enzymes. De novo synthesis of extracellular matrix molecules, however, is also required, and it has been shown that endothelial cells forming cords and tubes, for example, up-regulate type I collagen synthesis [19]. Other extracellular matrix molecules like laminin [25], fibronectin [17], thrombospondin [18] and SPARC [27] have been shown to modulate angiogenesis by influencing cell shape, cell adhesion and the availability of growth factors, respectively. Integrins of the α_1-subfamily and the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are considered to be involved in the signal cascade of matrix molecules during angiogenesis [5].

Proteoglycans of the extracellular matrix are also known to modulate interactions of cell surface receptors with their matrix ligands and to influence growth factor availability and function. The small proteoglycan biglycan is the principal chondroitin/dermatan sulfate proteoglycan synthesized by

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confluent cultured aortic endothelial cells [21], and it becomes up-regulated when endothelial cell migration is stimulated in vitro in response to monolayer wounding [22, 23]. Sprouting endothelial cells begin to express the related proteoglycan decorin [20], the most ubiquitously distributed small chondroitin/dermatan sulfate proteoglycan. This is of interest not only because decorin binds to type I collagen fibrils and regulates collagen fibrillogenesis [6] but also because of its growth regulatory properties. Decorin has been shown to suppress the growth of some tumor cells by activating the epidermal growth factor receptor [30] followed by the up-regulation of $p21^{Cip1/Waf1}$ [7], and it exerts an indirect effect on growth by complex formation with TGF-β [16].

In the present study we used a human endothelium-derived permanent cell line, EA.hy 926 [9], which had previously been used successfully in an in vitro model of angiogenesis [1]. In monolayer culture, these cells express biglycan but not decorin, even after stimulation by a variety of growth factors and cytokines [32]. In the present study we show that EA.hy 926 cells begin to produce decorin when they are maintained on top of or within collagen lattices, provided that they are kept in co-culture with fibroblasts. In co-culture, they form cords and tubes and escape apoptosis. Infection of endothelial cells with an adenovirus encoding for human decorin can substitute for the presence of fibroblasts.

Materials and methods

Immunological reagents

Monoclonal [41] and polyclonal, affinity-purified antibodies against human decorin and biglycan [15] and against rat decorin [47] have been described previously. Polyclonal antisera against type IV collagen and laminin-1 were kindly provided by Dr. H. Kleinman, National Institute of Dental Research, NIH, Bethesda, MD, USA. Polyclonal antibodies against von Willebrand factor-related antigen were obtained from Sigma, and secondary FITC- or Texas Red-labeled secondary antibodies (pig anti-rabbit IgG; goat anti-mouse IgG) were from DAKO Diagnostika, Hamburg, Germany.

Construction of Adv-DCN

The human decorin cDNA [24] was cloned into the adenovirus shuttle vector pAC-EF1. This vector is a derivative of pACCMV [11] and harbors the adenovirus serotype 5 sequences between 0.0 and 1.3 and between 9.3 and 17.0 map units. The region between map units 1.3 and 9.3 is replaced by the EF-1 promoter, the multicloning site and a BGH polyadenylation signal. A NotI site was added to the decorin cDNA by PCR using the primer pairs 5′-*CGCCAGTGTGCTGGAATTC*-3′ (5′-sequence at the multicloning site) and 5′-ATTAGCGGCCG-CTATAGAATAGGGCCCTCTAGA-3′ (reverse and complement with an added NotI site of the 3′-end of the multicloning site). After ligating the amplified cDNA into pAC-EF1, the integrity and orientation of the insert was verified by DNA sequence analysis. Plasmid DNA was purified by CsCl density gradient centrifugation and used to co-transfect 293 cells [2] with the pJM17 plasmid, which contains the adenovirus type 5 genome but lacks the E1 region and has a nonfunctional E3 gene [29]. When recombination between the two plasmids had occurred, the virus was subsequently plaque purified, grown in large quantity, and purified by 2 rounds of CsCl gradient centrifugation. The titer of the virus, determined by limiting dilution plaque assay, was 4×10^{10} plaque-forming units (pfu)/ml. In addition, the number of virus copies was determined by PCR with specific primers for human decorin (see below) and with adenovirus specific primers (sense: 5′-GTAGAGTCATAATCGTGCATCAGG-3′, antisense: 5′-TTTATAT-GGTACCGGGAGGTGGTG-3′). An insert-free adenovirus, Ad dl312, and a firefly luciferase gene-containing adenovirus which were used for control purposes, have been described before [33].

Cell culture

Cells of the permanent human endothelial cell line EA.hy 926 [9] were grown in DMEM (Life Technologies, Eggenstein, Germany) supplemented with 10 % fetal calf serum (BIOSPA, Wedel, Germany), antibiotics, 100 μM hypoxanthine, 0.4 μM aminopterine and 16 μM thymidine (Life Technologies). Rat fibroblasts were cultured in MEM which contained additionally non-essential amino acids.

Collagen gels were prepared according to Greve et al. [13], using 1.5 mg type I collagen from calf skin (Sigma, Deisenhofen, Germany) and 50000 rat skin fibroblasts in a final volume of 1.5 ml Waymouth MAB 87/3 medium per 3-cm hydrophobic plastic dish (Greiner, Solingen, Germany). The lattice was allowed to gel for 30 min at 37 °C. Thereafter, 500000 EA.hy 926 cells were seeded on top of the gel in a volume of 0.5 ml Waymouth medium. Alternatively, both fibroblasts and endothelial cells were incorporated simultaneously into the collagen gel. After 5 h at 37 °C the liquid medium was removed, the collagen lattices were washed twice with 2 ml each of serum-free Waymouth medium, and incubation continued in 2 ml of this serum-free medium. Half of the medium was replaced by fresh medium with 1 % heat-inactivated fetal calf serum every 6th day.

Viral infection of EA.hy 926 cells

If not otherwise stated, viral infection was performed as follows. EA.hy 926 cells which had been plated the day before (1×10^6 cells/25 cm^2-flask) were treated with 2×10^8 pfu of Adv-DCN or with one of the control virus constructs in 1.3 ml/25 cm^2-flask of DMEM containing 2 % heat-inactivated fetal calf serum. After 90 min of incubation 4 ml of standard medium per 25 cm^2-flask was added, and incubation continued for an additional period of 23 h. Subsequently, the cells were harvested for collagen lattice experiments as described below or subjected to metabolic labeling studies. Both control virus constructs yielded similar results.

Immunohistochemistry

Collagen gels were fixed with 4 % paraformaldehyde in 18 mM sodium phosphate/0.15 M NaCl, pH 7.4 (PBS) and embedded in paraffin. Sections of 6 μm thickness were placed on polylysine-coated glass slides, deparaffined with xylene and descending dilutions of ethanol, washed with PBS and briefly fixed again with 4 % paraformaldehyde. After further washes with PBS they were treated for 2 h at 37 °C with 0.1 U/ml of chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan) in this buffer. Nonspecific binding was blocked by incubation overnight at 4 °C with 5 % (w/v) bovine serum albumin (Serva, Heidelberg, Germany) and 10 % normal goat serum in PBS. The sections were then incubated with 1:50 dilutions of polyclonal antisera for 2 h at 37 °C. Preimmune sera from the same animals were used as controls. After 3 washes with PBS the slides were incubated for 2 h at 37 °C with a 1:2000 dilution of peroxidase-conjugated goat antibodies against rabbit IgG (Bio-Rad Laboratories, Munich, Germany). After four further washing steps antibody binding was detected with 0.75 mM diaminobenzidine and 0.015 % H_2O_2. Counter staining was performed with Giemsa stain.

Whole mount collagen gels were fixed on glass slides by drying followed by treatment with 4 % paraformaldehyde for 10 min at ambient temperature and with 70 % ethanol overnight at 4 °C and used for immunostaining. After washing with PBS and blocking of non-specific binding sites, immunostaining was carried out as described above except that 1:250 dilutions of the primary antisera were used.

Paraffin sections from granulomatous tissue around the anal fistula of a 46-years old patient were obtained through the courtesy of Dr. K. W. Schmid (Institute of Pathology, University of Münster). The sections were rehydrated as above and stained with monoclonal decorin antibodies (diluted 1:4), or with polyclonal antibodies against von Willebrand factor-related antigen (diluted 1:200) and biglycan (diluted 1:100), respectively. Secondary antibodies were diluted 1:30 (pig anti-rabbit IgG) or 1:50 (goat anti-mouse IgG). The sections were viewed by a Bio-Rad MRC 600 laser scanning microscope using the filters K1/K2 in case of double labeling and either BHS (FITC) or GHS (Texas Red) in single stains, respectively. The images were either pro-

cessed by LaserSharp software (Bio-Rad) or by Imaris software (Bitplane, Zurich, Switzerland).

TUNEL assays for apoptosis were performed according to Ramachandra and Studzinski [34] with minor alterations. Paraffin sections were rehydrated with xylene and descending concentrations of ethanol. The sections were kept in H_2O at 60 °C for 1 h and then equilibrated with 30 mM Tris/HCl buffer, pH 7.2, containing 140 mM sodium cacodylate and 1 mM cobalt chloride for 20 min at room temperature. DNA labeling was done in the same buffer upon addition of 62.5 U/ml terminal transferase and 3.75 nmol/ml biotin-16-ddUTP (Boehringer Mannheim, Mannheim, Germany). After 1 h at 37 °C the reaction was stopped. Nonspecific binding was blocked with 2 % bovine serum albumin and 4 % normal goat serum in PBS, and detection was achieved by treatment with a 1 : 1000 dilution of peroxidase-conjugated Extravidin (Sigma) for 30 min at 37 °C followed by incubation with substrate as described above. Counter staining was performed with 1 % methyl green in 20 % ethanol for 1 min and subsequent treatment with 70 % ethanol. The proportion of apoptotic (brown) nuclei was determined after evaluation of all nuclei observed in randomly selected microscopic fields (magnification: 400×).

Electron microscopy

Collagen lattices were examined by thin-section electron microscopy. After fixation with Karnovsky's fixative (5 % glutaraldehyde/4 % paraformaldehyde in 0.2 M sodium cacodylate buffer), followed by postfixation in 1.3 % OsO_4 in barbital buffer, pH 7.0, and dehydration in a graded ethanol series, they were transfered to propylene oxide and embedded in Epon 812. Ultrathin sections were mounted on 100 mesh copper grids without Formvar support films and stained with saturated uranyl acetate and lead citrate. Sections were examined and images recorded using a Philips 410 electron microscope operated at 60 kV.

Reverse transcription-polymerase chain reactions

Total RNA was isolated from EA.hy 926 cells and human fibroblasts cultured on plastic dishes (about 6×10^6 cells/dish) with the RNeasy Kit (Qiagen, Hilden, Germany). Reverse transcription-polymerase chain reactions (RT-PCR) were performed with the Titan RT-PCR system (Boehringer Mannheim) with minor modifications. Total RNA from fibroblasts or endothelial cells (600–700 ng) was incubated with primers for the human sequences (each 1 µM, final concentration) of biglycan (sense: 5′-GAGTAGCTGCTTTCGGTCC-3′, anti-sense: 5′-CAAGGCTCCAAATGCATGTG-3′) and decorin (sense: 5′-CGACTTCGGAGCCTCCCT-3′, anti-sense: 5′-GAATTACTTATAGTTTCCGAG-3′), and GAPDH (sense: 5′-GAAGGTCGGAGTCAACGGATTTGG-3′, anti-sense: 5′-CATGTGGGCCATGAGGTCCACCAC-3′), respectively, in a final volume of 50 µl. For reverse transcription the samples were incubated for 45 min at 50 °C and then heated for 2 min to 94 °C. For the PCR reaction 35 cycles were performed (denaturation, 94 °C, 1 min; annealing, 60 °C for biglycan and GAPDH/56 °C for decorin, 1 min; elongation, 72 °C, 1 min). The final elongation was at 72 °C for 7 min. Rat decorin mRNA could not be amplified under these conditions.

Five collagen gels were centrifuged to remove residual medium and lysed with the QIAshredder (Qiagen). Total RNA was purified with the RNeasy kit as described. The purification column was eluted with 35 µl of H_2O, and 5 µl of RNA were used for each RT-PCR. When no DNA was detected after RT-PCR on agarose gels stained with ethidium bromide, the denatured DNA was blotted onto Hybond filter membranes (Du Pont de Nemours, Bad Homburg, Germany) according to the Southern blot procedure and probed with a ^{32}P-labeled, random-primed cDNA for decorin [42] in 7 % SDS, 1 % BSA, 10 mM EDTA, 250 mM Na_2HPO_4, pH 7.2, at 65 °C for 2 h after prehybridization in the same buffer without probe. The blots were washed twice at the same temperature for 15 min each with 30 mM NaCl, 3 mM sodium citrate, pH 7.0, (1 × SSC) + 0,1 % SDS, twice for 15 min with 0.1 × SSC + 0.1 % SDS and exposed on Kodak X-Omat films with intensifying screens at −80 °C.

The RT-PCR for decorin was quantified by competition with an in vitro synthesized mRNA template from a decorin mutation (Del 7) which lacks 60 nucleotides in comparison to normal decorin cDNA and which was cloned into pcDNA3 [24]. A sense mRNA was transcribed with T7 RNA polymerase (Promega, Mannheim, Germany) from the insert of this plasmid after linearization with Dra III [42] and purified with the RNeasy kit. On the basis of the OD_{260} and the size of the mRNA the number of molecules was calculated, and a standard curve from 5×10^4 to 5×10^7 molecules was generated for RT-PCR. For quantification, the RT-PCR procedure was carried out in the presence of several concentrations of the mRNA standard. After agarose electrophoresis the resulting pattern of standard and sample bands was subjected to image analysis to calculate the standard concentration yielding the same amount of cDNA as the sample to be analyzed.

Other methods

Metabolic labeling with [^{35}S]sulfate and immunoprecipitation of secreted decorin followed by SDS-PAGE and fluorography were carried out as described [42]. Ethidium bromide stained bands obtained after RT-PCR and agarose gel electrophoresis were quantitated on a Omni Media XRS Scanner with the Whole Band Analyzer software (Bio Image™, version 3.2) from MWG Biotech (Ebersberg, Germany).

Results

Presence of decorin in capillaries of granulomatous tissue

Decorin expression was investigated in vivo by immunofluorescence labeling of human granulomatous tissue – a tissue, in which the formation of new capillaries can be anticipated. As expected, decorin was found in the loose connective tissue around the capillaries (Fig. 1A and B). In addition, the cytoplasm of some (Fig. 1A) but not all (Fig. 1B) endothelial cells of capillaries was also decorin positive. The endothelial localization was ascertained by double staining for decorin and von Willebrand factor-related antigen (Fig. 1A). Furthermore, endothelial cells stained positive for biglycan in a sometimes punctate manner which may possibly indicate the accumulation of the proteoglycan during intracellular transport in the Golgi apparatus (Fig. 1C).

Cultures of EA.hy 926 cells on plastic support

On the basis of these in vivo findings it appeared to be of interest to study decorin expression in more simple in vitro models. For this purpose we used EA.hy 926 cells which are immortalized derivatives of human umbilical vein endothelial cells. It was first investigated whether they can undergo phenotypical changes characteristic of their parental cells, for example sprouting, and whether these changes are accompanied by changes in the expression of small proteoglycans. Human umbilical vein endothelial cells start to synthesize and deposit decorin in a filamentous manner on top of the monolayer as soon as sprouting occurs, whereas biglycan remains diffusely distributed. EA.hy 926 cells show a cobblestone morphology when they are maintained in culture on a plastic support. Typical aspects of sprouting were not observed (results not shown). They express small quantities of biglycan as judged by Northern blot analysis, RT-PCR and immune precipitation with a monospecific antibody to biglycan (Fig. 2). Decorin mRNA and decorin core protein could not be detected by any of the assays employed.

It is known that sprouting can be triggered by certain growth factors and phorbol esters which promote angiogenesis.

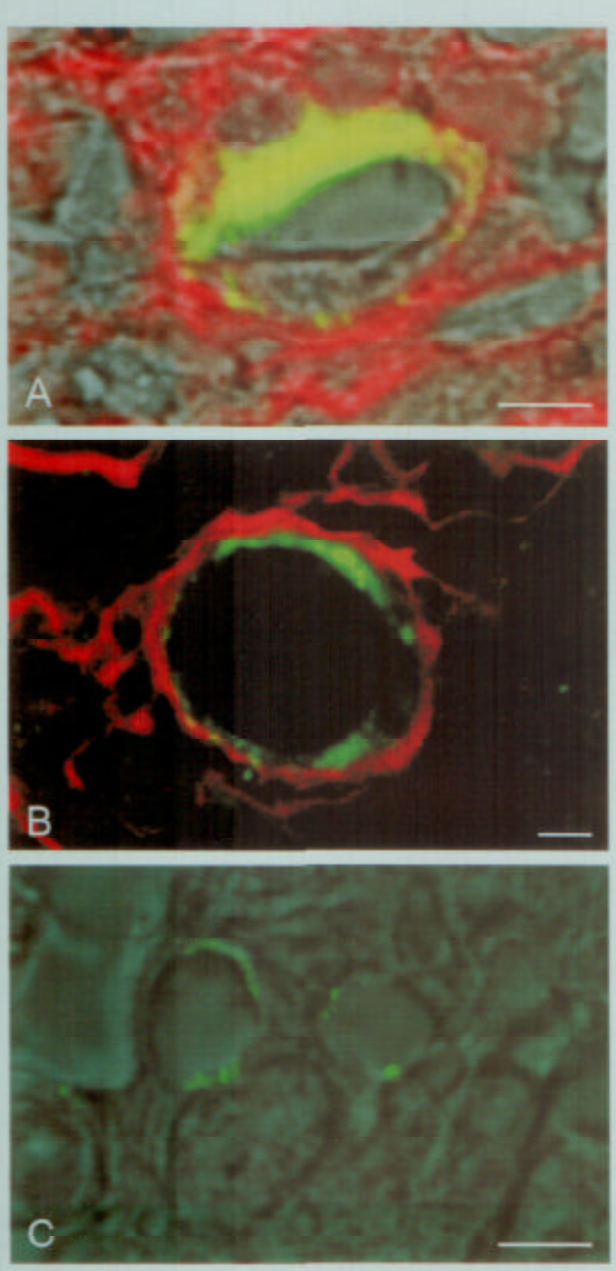

Fig. 1. Immunohistochemical detection of decorin in capillaries of granulomatous tissue. Granulomatous tissue was stained by immunofluorescence for decorin, von Willebrand factor-related antigen, and biglycan and viewed by laser scanning microscopy. A decorin/von Willebrand factor double staining is shown in panels **A** and **B**. In panel **A** the rhodamine channel (decorin) and the FITC channel (von Willebrand factor) are superimposed over the bright-field image. Decorin appears to be co-localized with von Willebrand factor in the endothelium of the capillary. In addition, it was also found in the surrounding loose connective tissue. In panel **B** decorin *(red)* is not present in the cytoplasm of endothelial cells. The endothelium is only stained for von Willebrand factor (*green*). In panel **C** immunofluorescence staining of biglycan is displayed. Biglycan appears in the endothelium of capillaries. The green fluorescence image was superimposed over the bright-field image. Bar = 5 µm.

EA.hy 926 cells in monolayer cultures were treated with the following agents to induce sprouting: fetal calf serum (10 %, v/v), the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (10 nM), bFGF (basic fibroblast growth factor, 2.9 nM), TGF-β1 (transforming growth factor-β1, 300 pM) and TNF-α (tumor necrosis factor-α, 2.9 nM) together with or without bFGF (2.9 nM). In contrast to the effect of those agents on early passage human arterial and venous endothelial cells, neither sprouting nor decorin synthesis could be induced in EA.hy 926 cells within 6 days (result not shown).

Cell cultures in floating collagen lattices

The culture of cells in three-dimensional matrices has often been employed for creating a more physiological environment to the cells than the usual monolayer cultures on plastic dishes. To apply this culture technique to EA.hy 926 cells, rat fibroblasts were first incorporated into a collagen lattice. After 30 min, when the collagen solution had gelled, endothelial cells were seeded on top of the floating gel, and the culture was continued with 1 % serum. Under these conditions endothelial cells assembled in cord-like structures and started to migrate into the interior of the gel where they formed lumina most often surrounded by a single layer of cells (Fig. 3). Tight junctions between adjacent cells were observed (Fig. 4). Thus, EA.hy 926 cells were able to assume a sprouting phenotype under appropriate experimental conditions.

The availability of species-specific antibodies for human and rat decorin, respectively, made possible to test separately for the deposition of fibroblast- and EA.hy 926 cell-derived decorin. It can be seen in Fig. 5 that after 6 or more days, the cords of endothelial cells were surrounded by human decorin. Rat decorin was diffusely distributed throughout the collagen gel and was absent from the cord-like structures seen on top of the lattice. As a modification of the co-culture model, EA.hy 926 cells were incorporated into the collagen gel together with rat fibroblasts. In this type of culture many lumina with protruding cells were seen. These lumina were also surrounded by human decorin. In the absence of fibroblasts no decorin and no typical cord-like structures could be detected. However, cavities were occasionally seen when EA.hy 926 cells alone were embedded into the collagen lattice.

In the collagen gel, EA.hy 926 cells started to synthesize laminin-1 and type IV collagen regardless of whether or not they were maintained in co-culture with fibroblasts. Positive immunoreactions for both proteins could be detected after 6 days inside the cells and around some cells. An analysis after 12 days revealed a patch-like accumulation of these basement membrane components (data not shown).

Quantification of decorin expression

To quantify the amount of decorin expressed by endothelial cells RT-PCRs were performed. In these experiments the amount of decorin mRNA synthesized by EA.hy 926 cells was compared to a known amount of internal standard mRNA of truncated decorin used as competitor. Bands of the same intensity were considered to originate from similar amounts of mRNA. After normalization of all the samples to the amount of the mRNA for GAPDH it could be shown that EA.hy 926 cells grown in a collagen gel without fibroblasts expressed after 12 days less than 7×10^3 copies of decorin mRNA per dish which was the limit of detection. In co-culture the expression was about 6×10^5 copies per dish, i.e. at least 100 times higher. In the absence of fibroblasts, the synthesis of decorin

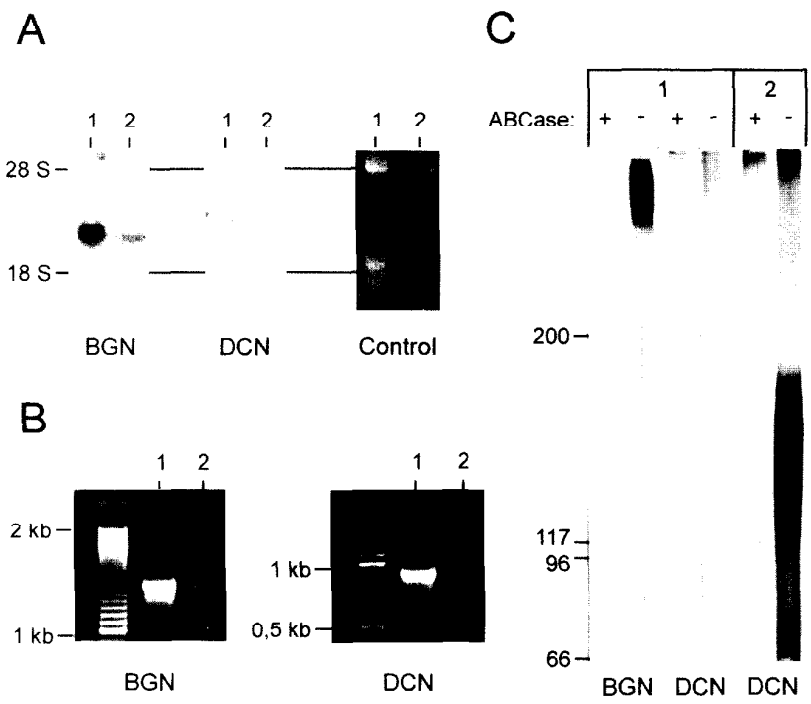

Fig. 2. EA.hy 926 cells synthesize biglycan but not decorin in monolayer culture. (**A**) Northern blot of EA.hy 926 cells with (*1*) 15 μg or (2) 5 μg total RNA hybridized with a full-length ^{32}P-labeled probe for biglycan (BGN) or decorin (DCN), respectively. As reference for standardization, the ethidium bromide-stained agarose gel before transfer to the nylon membrane is shown. (**B**) RT-PCR for human biglycan (BGN) and decorin (DCN). (*1*) Template mRNA (600 ng) from human skin fibroblasts; (2) template mRNA (700 ng) from EA.hy 926 cells. (**C**) Analysis of biglycan and decorin synthesis by EA.hy 926 cells. EA.hy 926 cells (*1*) and human skin fibroblasts (2) were labeled with [^{35}S]sulfate for 24 h prior to immunoprecipitation of secreted biglycan (BGN) and decorin (DCN), respectively. Samples were separated by SDS-PAGE (7% polyacrylamide) prior to or after digestion with chondroitin ABC lyase (ABCase) and visualized by fluorography.

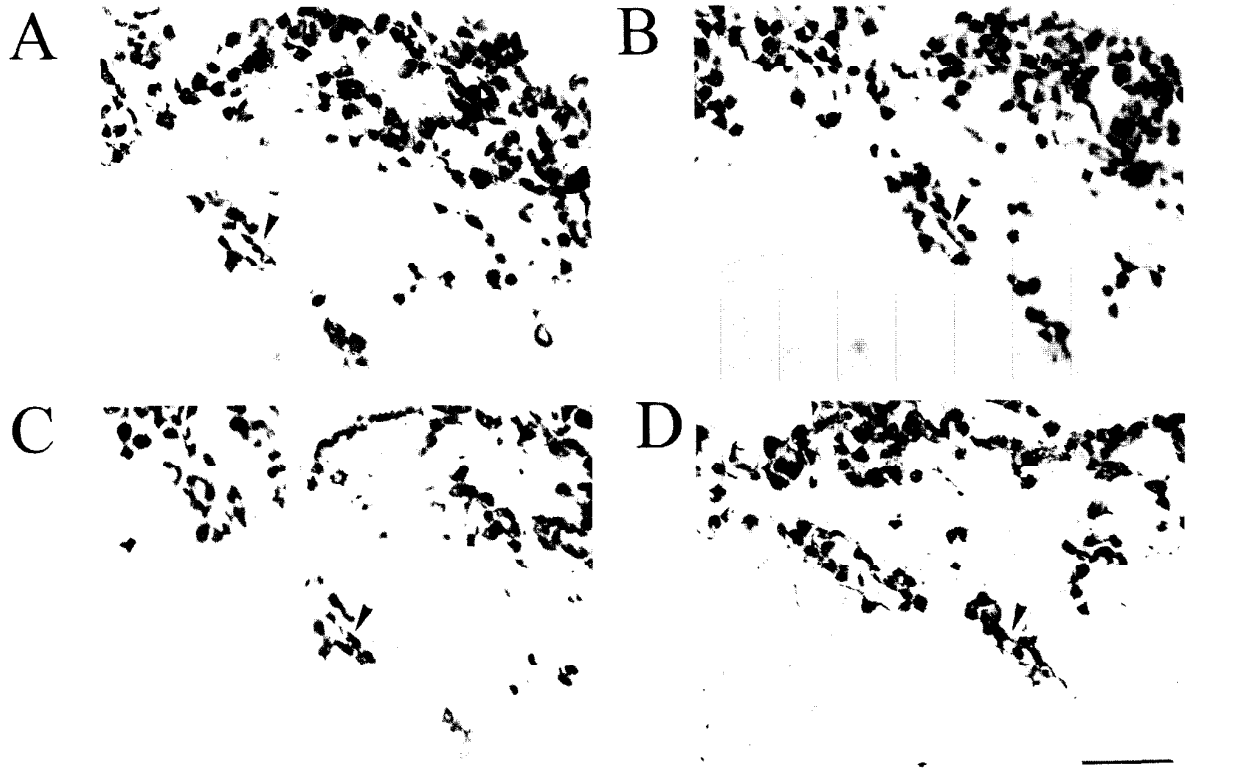

Fig. 3. Serial sections of EA.hy 926 cells grown on fibroblast-populated collagen lattices (**A–D**). EA.hy 926 cells were grown on top of the collagen gel for 12 days. *Arrowheads* point to capillary-like structures. Magnification: × 400. Bar = 30 μm.

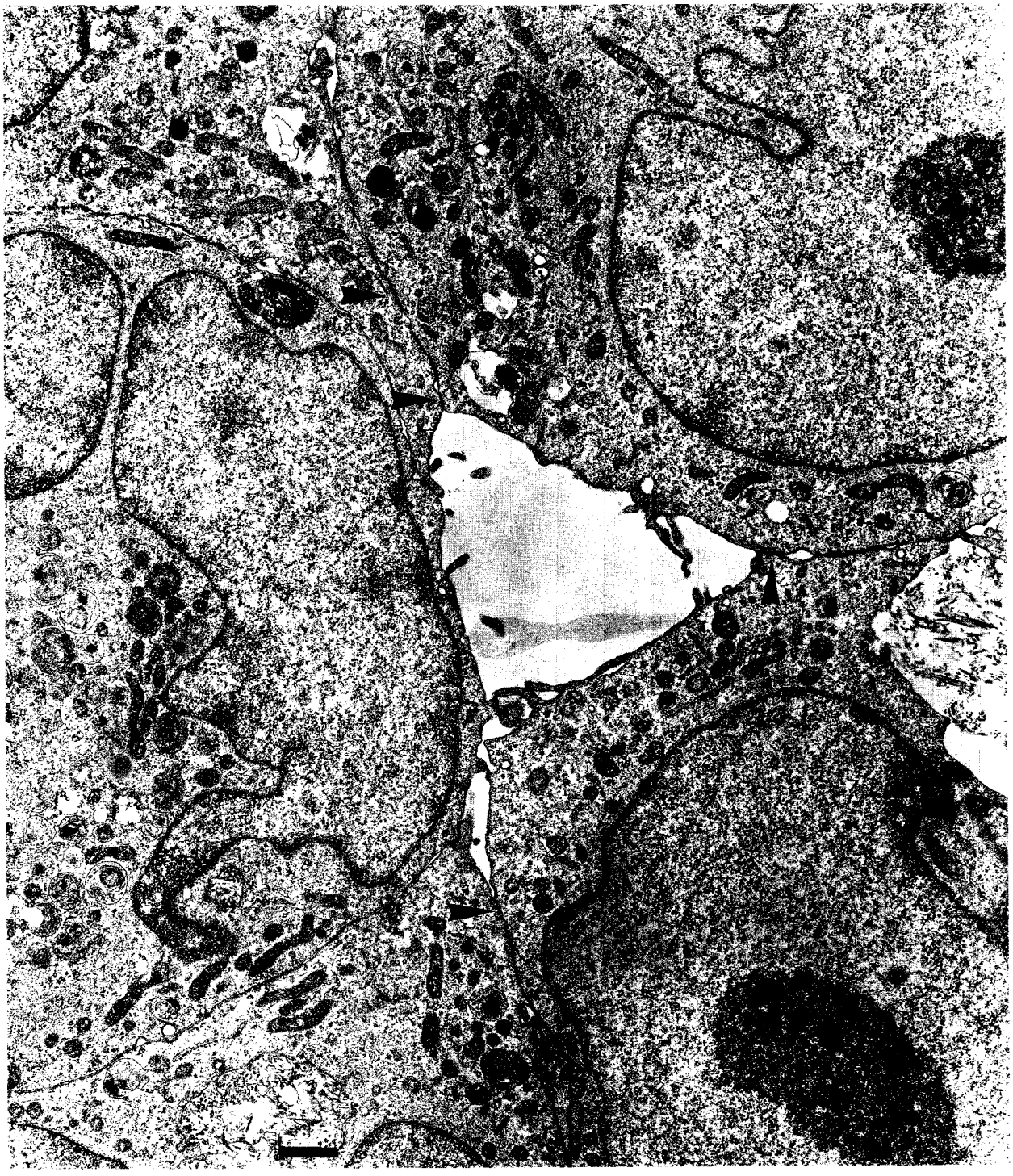

Fig. 4. Ultrastructural image of EA.hy 926 cells cultured for 12 days on top of a collagen lattice populated with rat fibroblasts. The cells are connected by extensive tight junctional strands (*arrowheads*). Bar = 1 μm.

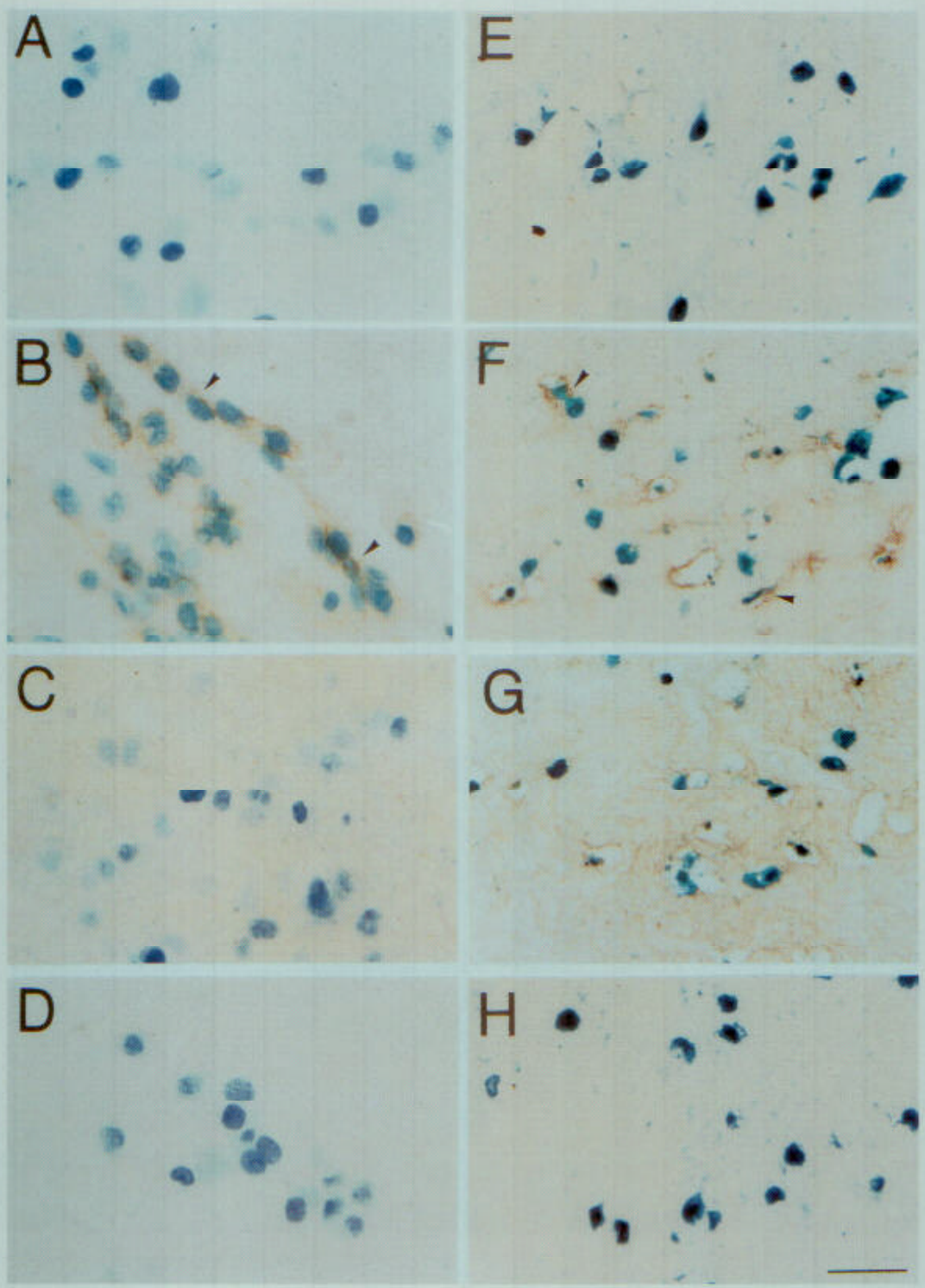

Fig. 5. Immunostaining with monospecific antibodies against human and rat decorin of EA.hy 926 cells grown either on top (**A–D**) or within (**E–H**) type I collagen gels. EA.hy 926 cells were grown for 6 days on top of or within collagen gels that either did not contain additional cells (**A** and **E**) or were populated with rat fibroblasts (**B, C, D** and **F, G, H**). Staining was for human decorin in **A, B, E,** and **F,** and for rat decorin in **C** and **G**. In **D** and **H** the first antibody was omitted. Note that only weak staining for rat decorin can be seen in **C** because only endothelial cells on the top of the lattice are displayed. Magnification: ×400. Bar = 50 μm.

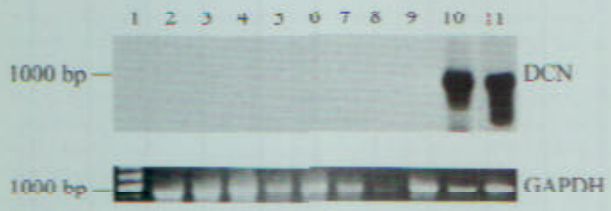

Fig. 6. RT-PCR for decorin after treatment of EA.hy 926 cells cultured in collagen lattices with different growth factors or in the continuous presence of serum. Total RNA was isolated from five collagen gels populated with EA.hy 926 cells for six days. The factors were added on the first day and on the fifth day again. For RT-PCR, RNA from 100000 EA.hy 926 cells in collagen gels was used for each experiment. *Upper panel:* Autoradiography of a Southern blot from the samples of the RT-PCR for decorin. The blot was hybridized with a full-length ^{32}P-labeled decorin probe (exposure time 16 h). *Lower panel:* Ethidium bromide-stained gels with samples from RT-PCR for GAPDH. *Lane 1*, molecular weight standards; *lanes 2* and 5, EA.hy 926 cells without growth factors; *lane 3*, 10 % fetal calf serum; *lane 4*, 2.9 nM TNF-α; *lane 6*, 300 pM TGF-β; *lane 7*, 2.9 nM bFGF; *lane 8*, 2.9 nM VEGF; *lane 9*, 3.3 nM EGF. As a control 600 ng RNA from human skin fibroblasts was used for RT-PCR (*lane 10*). Decorin expression in EA.hy 926 cells was induced by co-culture with rat fibroblasts for twelve days (*lane 11*). Note, that the primers used for RT-PCR were specific for human decorin and did not allow an amplification of rat decorin.

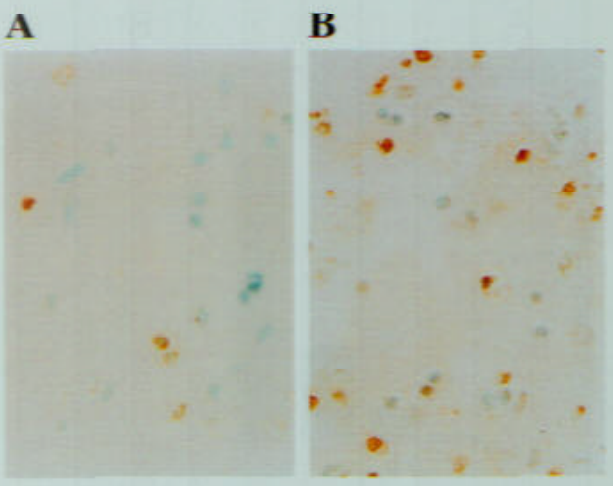

Fig. 7. TUNEL-assay for the detection of apoptotic cells. (**A**) EA.hy 926 were co-cultured with fibroblasts for 12 days, and in (**B**) endothelial cells were cultured alone for the same period of time. Cells undergoing apoptosis are characterized by brown nuclei when TUNEL assays are performed as described in the Materials and methods section. Magnification: ×400. Bar = 50 µm.

could not be promoted by growth factors known to be involved in angiogenesis (VEGF, 2.9 nM; bFGF, 2.9 nM; TGF-β, 300 pM; TNF-α, 2.9 nM; EGF, 3.3 nM; 10 % fetal calf serum). Even the attempt to detect minor amounts of PCR products by hybridization with a radioactively labeled probe for human decorin failed (Fig. 6). A combination of VEGF (2.9 nM) and bFGF (2.9 nM) was also ineffective (not shown).

Prevention of apoptosis in co-cultures

In the absence of fibroblasts, EA.hy 926 cells did not form cord-like structures and became disintegrated. Apoptotic cells were positively identified by the TUNEL assay (Fig. 7). Relatively few dying cells were seen after 6 days (Tab. I). However, after 12 days almost 60 % of the cells showed signs of apoptosis. In co-culture only 19 % of the cells were apoptotic. Several attempts failed to promote survival and cord formation of EA.hy 926 cells. Addition of bFGF (2.9 nM), VEGF (2.9 nM), EGF (3.3 nM), and TGF-β1 (300 pM) at the time of plating and again after 6 days in culture as well as the inclusion of 10 % fetal calf serum were ineffective although these factors are known to propagate the growth of endothelial cells in monolayer culture. The substitution of the growth medium with medium conditioned by fibroblast cultures for 3 days followed by dialysis against unused medium, was also without beneficial effect. When decorin (50 µg/ml) purified from cultured human skin fibroblasts or an equivalent dose of dermatan sulfate prepared from bovine aorta was included into the collagen gel at the time of lattice preparation, the extent of apoptosis was essentially unaffected (Tab. II). In addition, EA.hy 926 cells in a gel containing decorin failed to develop capillary-like structures (not shown).

Tab. I. Apoptosis of EA.hy 926 cells cultured in collagen lattices in the presence or absence of fibroblasts.

Experiment	Number of cells counted	% Apoptosis ± S.D.
EA.hy 926 cells (6 days in culture)	226	14.9 ± 5.7
EA.hy 926 cells + fibroblasts (6 days in culture)	226	13.8 ± 1.7$^{n.s.}$
EA.hy 926 cells (12 days in culture)	866	58.9 ± 5.6
EA.hy 926 cells + fibroblasts (12 days in culture)	628	19.3 ± 3.9***

$^{n.s.}$ $p > 0.05$; *** $p < 0.001$ compared with fibroblast free culture maintained for the same period of time (number of experiments, n = 3).

Tab. II. Effect of decorin expression and of exogenously added decorin and free chondroitin/dermatan sulfate glycosaminoglycan chains, respectively, on apoptosis of EA.hy 926 cells.

Treatment	Number of cells counted	Number of experiments	% Apoptosis ± S.D.
None	866	n = 4	58.9 ± 5.6
DCN	732	n = 4	55.2 ± 5.7$^{n.s.}$
CS/DS	868	n = 4	56.1 ± 3.1$^{n.s.}$
AdvDCN	881	n = 4	29.2 ± 7.6**
AdvCo	1030	n = 4	61.9 ± 8.9

** $p < 0.01$ compared to AdvCo; $^{n.s.}$ $p > 0.05$ compared to untreated culture. – Decorin (DCN) and chondroitin/dermatan sulfate (CS/DS) were added at a concentration of 50 µg/ml each at the beginning of the experiment, and the cells were maintained in the collagen lattice for 12 days. EA.hy 926 cells were infected with 200 pfu/cell of Adv-DCN or Adv-Co (Control) prior to culture in collagen lattices for 12 days.

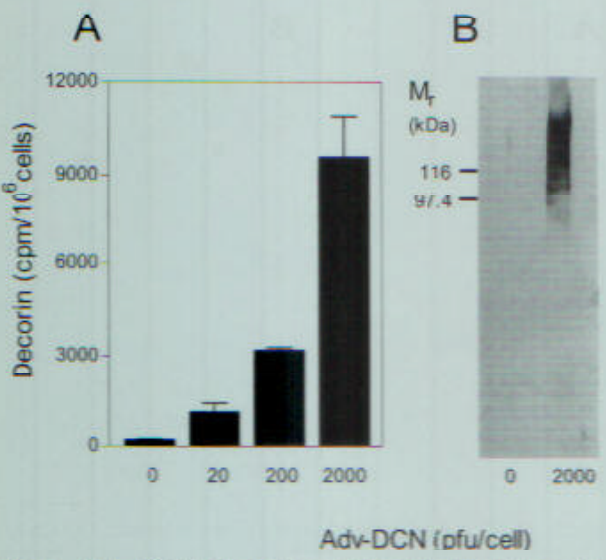

Fig. 8. EA.hy 926 cells express decorin after infection with recombinant decorin-encoding adenovirus (Adv-DCN). (**A**) EA.hy 926 cells were incubated with different doses of Adv-DCN and metabolically labeled from 24 h to 40 h after infection with [^{35}S]sulfate. Decorin was immunoprecipitated with a monospecific antibody and quantified by scintillation counting. Error bars indicate the standard deviation of triplicate experiments. (**B**) Western blot of decorin secreted by EA.hy 926 cells from 24 h to 72 h after infection with Adv-DCN compared to mockinfected cells.

Virus-mediated decorin expression

The experiments described above could not address the question whether or not there was a causal relationship between decorin expression on the one hand and cord formation and prevention of apoptosis on the other hand. A replication-deficient adenovirus was therefore constructed in which human decorin cDNA was under the control of the EF-1 promoter for constitutive expression of the proteoglycan. Treatment of monolayer cultures of EA.hy 926 cells with the virus led to a dose-dependent induction of decorin expression as shown by the incorporation of [^{35}S]sulfate into the proteoglycan and by Western blotting of conditioned media (Fig. 8). The episomal expression of decorin continued at a similar level for 6 days and declined to levels at the limit of detection during the following 6 days (not shown).

Virus-infected EA.hy 926 cells in collagen lattices

Upon infection of EA.hy 926 cells with decorin or control virus, the cells were incorporated into collagen lattices in the absence of fibroblasts and analyzed after 12 days of culture. As expected, cultures with Adv-DCN-infected cells stained positive for decorin. Most importantly, however, was the observation that capillary-like structures were formed (Fig 9) as reported above for co-cultures of uninfected EA.hy 926 cells and fibroblasts. Infection with a control virus was ineffective (Fig. 9). TUNEL assays indicated that in contrast to the infection with control virus the proportion of apoptotic cells was markedly reduced on application of Adv-DCN (Table II). In spite of this apoptosis-reducing effect which was observed in four independent experiments, the data (Tab. II) also indicate that the lowest percentage of apoptotic cells was measured in virus-free co-cultures of EA.hy 926 cells and fibroblasts.

Discussion

The results of the present investigation demonstrate that *(i)* endothelial cells in vivo express decorin in granulomatous tissues in which angiogenesis takes place, *(ii)* umbilical vein-derived endothelial cells (EA.hy 926 cells) form cord- and tube-like structures, express decorin, and escape apoptosis in vitro when they are co-cultured in collagen lattices with fibroblasts, *(iii)* decorin expression (mediated by infection of endo-

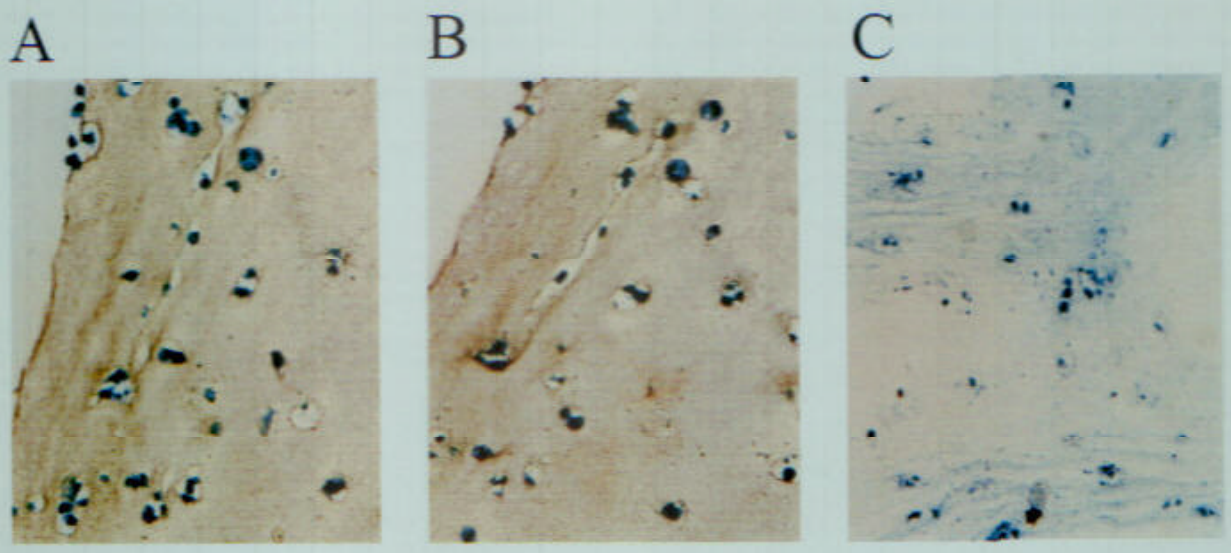

Fig. 9. Immunostaining for human decorin of EA.hy 926 cells maintained for 12 days in type I collagen lattices after infection with Adv-DCN or Adv-Co (Control). (**A, B**) Serial sections of a collagen gel populated with EA.hy 926 cells infected with Adv-DCN and (**C**) section of a collagen gel containing EA.hy 926 cells infected with a similar dose of Adv-Co. Magnification: ×200. Bar = 100 µm.

thelial cells with a replication-deficient adenovirus) allows the cells independently from the presence of fibroblasts to form tube-like structures and leads to a significant reduction of apoptosis. These data can be interpreted by two not necessarily exclusive hypotheses. First, decorin may be considered to be a true angiogenic factor. Indeed, it fulfills several criteria of such a factor [35]. It is present when angiogenesis occurs and absent in the endothelium of resting capillaries [4], and there is a decorin-specific receptor in the plasma membrane of endothelial cells [12]. However, a correlation between decorin expression and the quantity of formed blood vessels could not be elaborated with the present methodology. It is also noteworthy that decorin knock-out mice do not exhibit an obvious anomaly of their vasculature [6]. This finding, however, does not prove that decorin is not involved in angiogenesis. Since there are many examples for the redundant expression of functionally related proteins, the absence of one of them is not necessarily linked with an abnormal phenotype [10]. The second hypothesis postulates that it is the decorin-mediated prevention of apoptosis in the culture model that allows the cells to survive for the development of tube-like structures. It had been shown before that angiogenic stimuli like those provided by FGF-2 [39] are essential for the survival of endothelial cells in collagen lattices and that fibroblasts prolonged the life span of microvessels in a 3-dimensional collagen gel [44]. The agent(s) being responsible for the latter effect had not been investigated. However, it had been shown, for example, that the cell membrane proteoglycan syndecan-1 induced apoptosis of myeloma cells [8] whereas an apoptosis-preventing effect of a proteoglycan had not yet been reported. An indirect effect of decorin on apoptosis can also be envisaged. For example, TGF-β induces apoptosis of gastric cancer cells [49], and matrix-bound decorin could work by withdrawing the cytokine from the signalling receptors of the cells [43]. Nevertheless, the second hypothesis appears to be less likely, since the manifestation of the effect of decorin on apoptosis required more than 6 days of culture within the lattice whereas an effect on tube and cord formation was seen before.

The mechanism of action of decorin on angiogenesis is not yet known. Collagen gels and complex extracellular matrix substrates induce differentiation by several cooperating mechanisms, including alterations of cell shape by tensile forces. Decorin may delay the retraction of collagen lattices [3] and may therefore influence the mechanochemical switching between growth and differentiation during angiogenesis. Related to these observations may be the findings that decorin interacts with the cell-binding and heparin-binding domains of fibronectin [28, 41] and with several domains of thrombospondin [46]. Since fibronectin [17] and thrombospondin [31] themselves play an important role during angiogenesis and since decorin delays cell adhesion to these substrates [45, 46], it is likely that decorin is a further component in the list of functionally relevant modulators of endothelial cell adhesion to extracellular matrix molecules.

Decorin may also have direct effects on growth factor activities and on the progression through the cell cycle. As mentioned above it forms complexes with TGF-β [43, 48] which has been shown to be involved in several steps of angiogenesis [26]. Additionally, decorin seems to be directly involved in growth control at least of selected types of cells [38], and it does so by up-regulation of $p21^{Cip1/Waf1}$ [7], an inhibitor of cyclin-dependent kinases, via the up-regulation of the EGF receptor [30]. Preliminary investigations show indeed that the $p21^{Cip1/Waf1}$ mRNA levels of EA.hy 926 cells were up-regulated as well by co-culture with fibroblasts as by infection with the decorin adenovirus (D. Fastermann and E. Schönherr, unpublished result). The importance of this phenomenon for tube formation and prevention of apoptosis remains to be investigated.

Considering the mechanism of action of decorin it is noteworthy that the inclusion of purified decorin into the collagen lattice at the beginning of the experiment did not enable the cells to form cords and tubes and to escape apoptosis. It is likely that the exogenously added decorin became bound to collagen fibrils before the cells spread and formed contacts with themselves. Adding decorin at later stages appeared inappropriate to study its effect on collagen-embedded cells since it does not diffuse through collagen lattices within the time period of the experiments [13]. Paracrine acting factor(s) secreted by fibroblasts may not only induce decorin but may have additional effects on the expression of other genes. On the other hand, the infection with a decorin-encoding adenovirus suggests that the onset of decorin production is sufficient to initiate the biological response. It is not known whether or not the expression of decorin correlates with the expression of other, unrelated genes. However, it had been shown before that the exogenous addition of relatively high concentrations of decorin (10 μg/ml) to tumor cells in monolayer led to an up-regulation of $p21^{Cip1/Waf1}$ via an interaction with the EGF receptor, indicating that decorin is indeed acting from the outside of the cell [30]. Newly synthesized decorin follows a secretion-recapture pathway in fibroblasts [40]. Under the special condition of cells cultured in a collagen lattice it may well be that sufficient concentrations for a signalling receptor on the cell membrane can be achieved only in an autocrine manner, i.e. by decorin-producing endothelial cells themselves.

In summary, our data provide evidence for the involvement of decorin during vessel formation in vivo and in an in vitro model of angiogenesis. The step(s) in the complex cascade of events during angiogenesis in which decorin may be involved remain to be determined.

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